

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
28 June 2001 (28.06.2001)

PCT

(10) International Publication Number  
**WO 01/46213 A2**

(51) International Patent Classification<sup>7</sup>: C07H 21/00

Klosterneuburg (AT). HILLER, Reinhard [AT/AT]; Löhner-  
nergasse 8, A-1120 Vienna (AT).

(21) International Application Number: PCT/EP00/13099

(74) Agents: SCHOHE, Stefan et al.; Boehmert & Boehmert,  
Franz-Joseph-Strasse 38, D-80801 München (DE).

(22) International Filing Date:  
21 December 2000 (21.12.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
99125484.8 21 December 1999 (21.12.1999) EP  
99125485.5 21 December 1999 (21.12.1999) EP  
60/211,217 13 June 2000 (13.06.2000) US  
60/211,209 13 June 2000 (13.06.2000) US

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,  
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,  
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,  
LS, LT, LU, LV, MA, MG, MK, MN, MW, MX, MZ, NO,  
NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR,  
TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(71) Applicant (*for all designated States except US*): LION  
BIOSCIENCE AG [DE/DE]; Im Neuenheimer Feld 515,  
D-69120 Heidelberg (DE).

**Published:**

— *Without international search report and to be republished  
upon receipt of that report.*

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): HUBER, Martin  
[AT/AT]; Rennweg 84, A-1030 Vienna (AT). SCHMIDT,  
Wolfgang [AT/AT]; Hetzgasse 12, A-1030 Vienna (AT).  
MÜLLER, Manfred [AT/AT]; Schömergasse 30, A-3400

*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*



WO 01/46213 A2

(54) Title: COMPOUND COMPRISING A PEPTIDE MOIETY AND AN ORGANO-SILANE MOIETY.

(57) Abstract: The present invention concerns a compound comprising a biomolecule moiety and an organo-silane moiety, as well as a process for the synthesis thereof. The invention also concerns a support comprising the biomolecule moiety with the organo-silane moiety, wherein the biomolecule moiety is attached to the support through the organo-silane moiety. The invention also concerns a process for a nucleic acid synthesis reaction making use of the biomolecule moiety with the organo-silane moiety as well as uses of the novel compound. The invention in addition concerns a kit comprising the compound comprising a biomolecule moiety and an organo-silane moiety.

“Compound Comprising a Peptide Moiety and an Organo-silane Moiety”  
Description

Background of the Invention

Genetic analysis often involves analysis of the nucleic acid sequence, structure or composition of a given organism or sample. Frequently, such analysis incorporates the step of, or requires nucleic acid amplification. One of the well known methods for nucleic acid amplification is the "PCR", or polymerase chain reaction method also disclosed in US 4,683,195 and US 4,683,202. Here, a nucleic acid sample serves as a template for a polymerase dependant *in-vitro* replication starting from two separate primers. Polymerases are enzymes capable of synthesising RNA or DNA making use of RNA or DNA as a template. Often times the analysis is performed on RNA (ribonucleic acid), here amplification additionally requires an enzymatic reverse transcription into DNA (deoxyribonucleic acid), but equally often on DNA. PCR is becoming powerful tool in diagnostics. PCR kits are becoming available for the detection and analysis of various pathogenic organisms as well *e.g.* mutant alleles of human genes.

PCR is mostly performed *in-vitro*, *i.e.* in a tube whereby the components are mostly supplied in liquid format. Alternatively, one or more of the components, these usually being, a polymerase, a buffer, a template, two or more oligonucleotides, may be bound to some form of a solid-phase.

One very common problem with the PCR being performed in a standard non solid-phase format is the limitation with respect to the number of primer pairs that may be used simultaneously in one reaction. In contrast a solid-phase set-up would theoretically enable the use of tens to thousands of primer pairs.

A problem with such an approach is the efficiency of the reaction, thus also the product yield during amplification is poor and consequently the reliability of the entire process. Often these problems are associated with the fact that the reaction conditions *i.e.* the availability of the primer in the reaction are not optimal. One may envision that the 3' OH ends of the oligonucleotides are not available due to the fact that parts of the oligonucleotide are bound to the support. Such immobilised single-stranded DNAs which have conventionally been used are prepared by binding a single-stranded DNA at the terminal molecule or a suitable functional group introduced into the molecule. However, such conventional method has a drawback in that it is impossible to bind the single-stranded DNA to the carrier only at its terminal molecule by the use of the conventional method because an existing amino or hydroxy group, or other functional group

artificially introduced, on the nucleotide molecules other than terminal also participates in the binding with the carrier. The immobilised DNA obtained with conventional methods is one in which the DNA molecules are bonded to the support at various sites of the strand. As will be recognised by those skilled in the art, such a molecule is poorly suited for providing sufficient experimental results in any aspects.

A further problem is that the molecules bound to the support are sterically hindered by the support itself from taking part in *e.g.* enzymatic reactions. EP0787205 discloses the use of linker between an oligonucleotide and the solid-support. However, the primary problem is not addressed here. The primers on a solid-support are not freely available in the reaction.

It is known to bind DNA primer to the support via reactive groups. Such reactive groups have been *e.g.* amino groups. It is known in the art that such groups are very unstable, consequently when a given primer pair is arrayed on a support wherein the terminal group of the primers are *e.g.* amino groups it is to be expected, based on the instability of the groups, that after arraying the primers in each pair will not be present equimolar manner.

It is therefore an object of the present invention to provide for a process, means and substances that lead to a high efficiency in in-vitro solid-phase enzymatic, like nucleic acid synthesis reactions or epitope-antibody reactions, thus also to a higher product yield and consequently a higher reliability of such reactions.

It is further an object of the present invention to provide for a process, means and substances to be used in in-vitro solid-phase solid-phase enzymatic, like nucleic acid synthesis reactions or epitope-antibody reactions, that are suited for providing good experimental results in many aspects such as, equimolarity of oligonucleotide amount, reduction of background signal, and reduction of falsely synthesised products.

It is a further object within the concept of the invention to provide for novel molecules capable of solving the above problems. Thus it is *e.g.* an object of the present invention to provide for molecules that are readily available in a DNA synthesis reaction and not sterically hindered by *e.g.* a solid-support or the like.

It is also an object within the concept of the invention to provide for processes for making the molecules according to the invention.

It is a further object within the concept of the invention to provide for a kit comprising one or more of the molecules according to the invention.

Equally it is an object of the present invention to provide for a kit comprising compounds necessary for performing the process according to the invention.

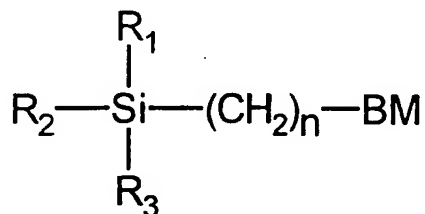
Further objects of the invention are apparent to the skilled person from the specification.

### Summary of the Invention

The objects of the present invention are accomplished by providing for a compound with novel characteristics which may be used in solid phase enzymatic reactions, a processes for making this compound, as well as kits containing a compound according to the invention for use in processes according to the invention as well as other processes.

The object of the present invention was accomplished by providing for a compound comprising a biomolecule moiety and an organo-silane moiety as represented in formula 1

formula 1:



wherein,  $\text{R}_1$ ,  $\text{R}_2$ , and  $\text{R}_3$  are identical or different alkoxy groups, wherein alkoxy refers to groups of the general formula  $-\text{OR}$ , wherein  $\text{R}$  is an alkyl rest, and "BM" represents a biomolecule moiety selected from the group comprising one or more amino acids, peptides and proteins and derivatives thereof and wherein,  $n$  is an integer from 0 to 18.

This novel compound has surprisingly shown unexpected results when compared to similar molecules previously used in solid-phase enzymatic reactions with respect to but not limited to the following effects, its adsorption capacity to a solid-support, its availability in enzymatic reactions, thus its contribution to the efficiency of e.g. solid-phase nucleic acid synthesis reactions or epitope-antibody reactions.

The alkoxy groups  $R_1$ ,  $R_2$ , and  $R_3$  may, e.g. by methoxy, ethoxy or the like. Within the scope of the invention are organo-silane moieties comprising mixtures of different alkoxy groups. For example,  $R_1$  may be a methoxy,  $R_2$  an ethoxy and  $R_3$  a methoxy. The alkoxy groups  $R_1$ ,  $R_2$ , and  $R_3$  may equally well be identical. The skilled artisan is credited with the ability to discern alternative combinations which shall be within the scope of the invention.

Herein, solid phase reactions and solid-support reactions are used with equal meaning and shall be understood as such reactions in which one or more compounds is attached to a solid matter of any given shape or chemical structure.

Herein, a biomolecule is to be understood as any molecule which shows enzymatic activity, which acts as a probe in molecular analysis or which is the target of an enzymatic activity.

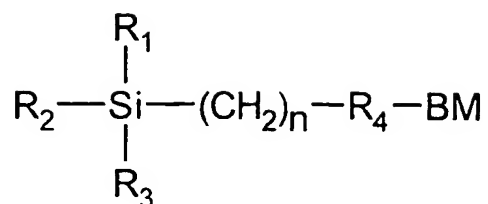
Also, one or more amino acids, peptides as well as proteins may be represented by the term biomolecules "BM" within the scope of the invention.

The compound according to the invention comprises an organo-silane as well as a biomolecule. It is obvious to one skilled in the art, that these two moieties may but must not be connected through one or more methylene groups. The compound according to the invention may thus also comprise the organo-silane group which is directly coupled to the methylene group.

In a preferred embodiment of the compound according to the invention however, the organo-silane group is bound to between 1 and 18 methylene groups which are bound to the biomolecule.

The inventors have found that synthesis of the compound according to the invention is facilitated if the compound according to the invention further comprises a linking moiety  $R_4$  interpost between the organo-silane moiety and the biomolecule moiety as represented by formula 2

formula 2:



wherein,  $\text{R}_1$ ,  $\text{R}_2$ , and  $\text{R}_3$  are identical or different alkoxy groups and BM represents the biomolecule moiety or a derivative thereof,  $n$  is an integer from 0 to 18 and wherein,  $\text{R}_4$  represents the linking moiety.

The compound according to the invention is preferentially synthesised with the aid of homo- or hetero-bifunctional groups. These groups are used to specifically connect a methyl group or alternatively the organo-silane with the biomolecule. These groups result in the linking moiety  $\text{R}_4$  after reacting. Thus, such a linking moiety within the scope of the invention is to be understood as any moiety stemming from a homo- or hetero-bifunctional group after having reacted with an organo-silane and a biomolecule.

The inventors have found that the organo-silane reacts both with other compounds according to the invention well, which may be desirable as outlined below as well as with various solid supports well if  $\text{R}_1$ ,  $\text{R}_2$ , and  $\text{R}_3$  are each methoxy groups. Thus in a preferred embodiment of the invention  $\text{R}_1$ ,  $\text{R}_2$ , and  $\text{R}_3$  are each methoxy groups.

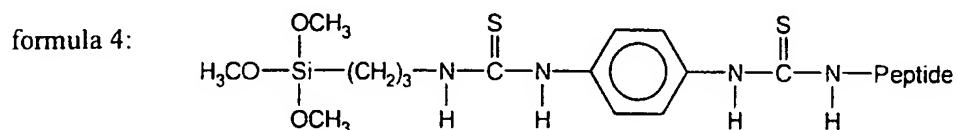
The biomolecule is preferentially coupled via a bifunctional linking moiety  $\text{R}_4$  to the organo-silane. It has been found that there are particularly suited bifunctional linking reagents for accomplishing this. Such bifunctional linking reagents may be selected from the group comprising arylenediisothiocyanate, alkylenediisothiocyanate, bis-N-hydroxy-succinimidylesters, hexamethylenediisocyanate and N-( $\gamma$ -maleimidobutyryloxy)succinimide ester.

Hence, once the bifunctional linking reagent has reacted with the adapter molecule "AM" or alternatively directly with the biomolecule "BM" at its first reactive group and the organo-silane at its second reactive group a linking moiety  $\text{R}_4$  is present in the compound according to the inven-

tion. In a preferred embodiment of the invention  $R_4$  is selected from the group comprising arylene(bisthiourea) and alkylene(bisthiourea).

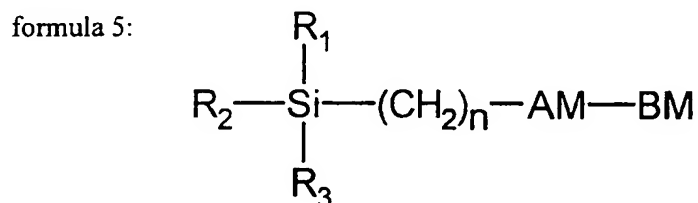
In a preferred embodiment of the compound according to the invention the linking molecule  $R_4$  is phenylenebisthiourea.

The inventors have found that a very particular compound according to the invention is easy to synthesise and shows excellent results in solid-phase experiments. This compound is represented by formula 4



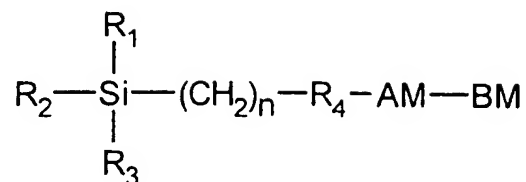
The compound comprises a biomolecule moiety, *i.e.* a peptide moiety, a linking moiety as well as an organo-silane moiety.

The compound according to the invention may further comprises an adapter moiety interposed between the organo-silane moiety and the biomolecule moiety where said compound is represented by formula 5,



or alternatively the compound further comprises an adapter moiety interposed between the linking moiety and the biomolecule moiety where said compound is represented by formula 5 A,

formula 5 A:



wherein, R<sub>1</sub>, R<sub>2</sub>, and R<sub>3</sub> are each and independently alkoxy groups, BM represents the biomolecule moiety or a derivative thereof, n is an integer from 0 to 18, R<sub>4</sub> represents the linking moiety and AM represents the adapter moiety.

In a preferred embodiment of the invention the adapter moiety "AM" is chosen from the group comprising -(CH<sub>2</sub>)<sub>n</sub> and -[(CH<sub>2</sub>)<sub>2</sub>O]<sub>n</sub> wherein n is an integer from 0 to 18.

In a preferred embodiment the biomolecule moiety is linked to the linking moiety or the to the adapter moiety via its N-terminal or its carboxy-terminal end.

The peptides can be coupled to the silanes in a variety of ways. One method is to couple the peptides via a disulfide bond which could be cleavable, e.g. with the thiol side chain of Cys in proteins. Other methods include the maleimide-hinge technique, the succinimide technique or the like where the protein to be bound is, for example, an antibody.

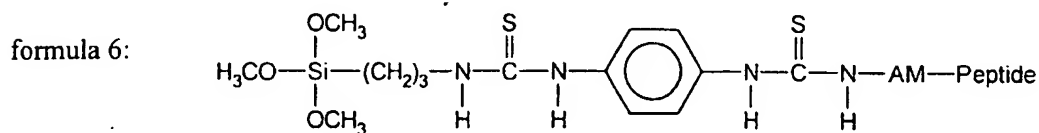
Further, organic and aqueous solutions of gamma-aminopropyltriethoxysilane (APS) can be used in order to generate a number of reactive NH<sub>2</sub> groups available for coupling to protein. The protein can be coupled to the silane by formation of Schiff's base linkages via glutaraldehyde. Another suited substance is trimethylchlorosilane

Other methods are based on the covalent cross-linking of the enzymes to bifunctional resins containing a secondary amino and thiol groups, e.g. in a coupling reaction with the imidoester dimethyl pimelimidate hydrochloride. Covalent immobilization of functional proteins on silica substrates can be performed using thiol-terminal silanes and heterobifunctional cross-linkers.



Immobilization can further be performed directly via silanes carrying alkyl moieties with terminal carboxylic groups. The enzyme can also be covalently attached to phospholipid-bound silanes through the terminal carboxyl moiety on the sn-2 acyl chain of the lipid.

In a particularly preferred embodiment of the invention the compound is represented by formula 6



In a particularly preferred embodiment according to the invention, peptide is an antibody or a functional part thereof. The antibody can be a monoclonal antibody, a humanized antibody or an scFv-fragment.

The inventors have found that the synthesis of the compound according to the invention is greatly facilitated if the biomolecule comprises a reactive group on a separate moiety which enables the binding to  $\text{R}_4$  or alternatively to the organo-silane. In the case of nucleic acids and oligonucleotides in particular such an adapter molecule may be characterised by formula 8,

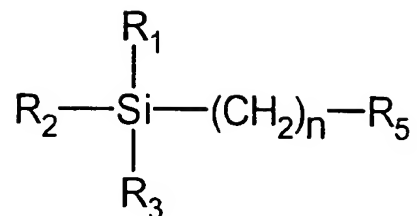
formula 8:



wherein  $\text{R}_6$  is selected from the group comprising cyanoethylphosphoramidites, Z is selected from the group comprising  $-\text{NH}_2$ ,  $-\text{SH}$  and, n is an integer from 0 to 18.

The invention also covers a process for the synthesis of a compound as disclosed above. In this process, an organo-silane is reacted with a biomolecule BM wherein, the organo-silane is represented by formula 7:

formula 7:



wherein,  $\text{R}_1$ ,  $\text{R}_2$ , and  $\text{R}_3$  are each and independently an alkoxy group,  $\text{R}_5$  is selected from the group comprising  $-\text{NH}_2$  (-amino),  $-\text{SH}$  (-sulfhydryl),  $-\text{NCO}$  (-cyanato),  $-\text{NHS}$  ester (hydroxysuccinimidylester, -acrylate) and  $n$  is an integer from 0 to 18.

The inventors have found that the above process is facilitated if prior to the reaction between the organo-silane and the biomolecule, BM is reacted with an adapter molecule AM and subsequently reacted with the organo-silane. One reason for this is simply that *e.g.* if the biomolecule is a peptide such an adapter molecule may be coupled during on-line synthesis.

It should be noted that the biomolecule may be initially reacted with a linking molecule and subsequently reacted with the organo-silane, or alternatively the organo-silane is reacted with the linking molecule and subsequently reacted with the biomolecule, wherein the linking molecule is a bifunctional reagent.

Alternatively, (i) a biomolecule is reacted with an adapter molecule resulting in reaction product A; reaction product A is reacted with a linking molecule resulting in reaction product B, and reaction product B is reacted with an organo-silane or alternatively, (ii) a biomolecule is reacted with an adapter molecule resulting in reaction product A; a linking molecule is reacted with an organo-silane resulting in reaction product C and reaction product A and C are reacted or alternatively, (iii) an adapter molecule is reacted with a linking molecule resulting in reaction product D, the reaction product D is reacted with the biomolecule resulting in reaction product B and reaction product B is reacted with an organo-silane or alternatively, (iv) an adapter molecule is reacted with a linking molecule resulting in reaction product D, the reaction product D is reacted with an organo-silane resulting in reaction product E and reaction product E is reacted with a biomolecule.

In a preferred embodiment in the process according to the invention the biomolecule BM is reacted with a linking molecule  $R_4$  and subsequently reacted with the organo-silane or alternatively, the organo-silane is reacted with the linking molecule  $R_4$  and subsequently reacted with the biomolecule BM wherein, the linking molecule  $R_4$  is a bifunctional reagent. Although the process according to the invention does not require such a linking molecule  $R_4$  the inventors find this to be advantageous.

While one skilled in the art will come up with various adapter molecules and thus the invention shall not be limited by the following example, the inventors have found the adapter molecule AM as represented by formula 8 is preferred.

formula 8:



Here,  $R_6$  is selected from the group comprising cyanoethylphosphoramidites, Z is selected from the group comprising  $-NH_2$ ,  $-SH$ ,  $-PO_4$ ,  $-COOH$ ,  $-I$  and, n is an integer from 0 to 18.

As outlined above it is preferred that the linking molecule is a bifunctional reagent, *i.e.* a coupling reagent with two reactive groups. In a preferred embodiment of the process according to the invention the linking molecule  $R_4$  is selected from the group comprising arylenediisothiocyanate, alkylenediisothiocyanate, bis-N-hydroxy-succinimidylesters, hexamethylenediisocyanate and N-( $\gamma$ -maleimidobutyryloxy)succinimide ester. It is evident that these are preferred examples and one skilled in the art may find other possible bifunctional reagents which are equally within the scope of the invention.

In a preferred embodiment the linking molecule  $R_4$  is 1,4-phenylene diisothiocyanate.

The inventors have coupled the compound according to the invention to glass supports (see example X and Y) and performed solid-support nucleic acid synthesis reactions.

This has drastic implications for the use of the compound according to the invention. Only to name a few of the advantages this incurs *e.g.* the background reduction will make it possible to perform solid-phase quantification experiments much more precisely, the bottom detection limit of analytes will drop, thus more precise results will be obtainable in various fields where the compound according to the invention finds applications.

The compound according to the invention is preferentially used in solid-phase reactions here the compound may be bound to substances chosen from the group comprising nitrocellulose, nylon, controlled-pore glass beads (CPG), polystyrene, activated dextran, modified polystyrene, styrene-acrylnitril-copolymers, polycarbonate, cellulose, polyamide and glass.

The inventors have bound a compound according to the invention to a support. In preferred embodiment such a support is glass. This may be done simply by incubating a clean glass slide with the compound comprising the organo-silane moiety and the biomolecule moiety. Thus, supports may be obtained comprising the compound according to the invention.

The inventors have astonishingly found that a support comprising a compound according to the invention exhibits a coating density of at least 1 pmol of biomolecule per  $\text{mm}^2$ , often 10 pmol of biomolecule per  $\text{mm}^2$  up to 80 pmol of biomolecule per  $\text{mm}^2$  and even higher. These high figures are not achievable when applying prior-art technology.

In a preferred embodiment the compound is used with glass although it may also be used in combination with any other solid-support. Such glass may be a glass slide, as used *e.g.* for microscopy, glass vessels or containers, glass fibers, glass beads or other -Si comprising glass entities.

The compound according to the invention may be spotted onto, pipetted onto, sprayed onto or otherwise brought onto such a glass support. Possible methods are, application by means of a needle, capillary, dispenser and piezo pipette is preferable, *e.g.* an apparatus similar to the kind known for ink jet printers.

The compound according to the invention may be used in various ways some of which shall be mentioned here. The compound is particularly suited for nucleic acid hybridization or synthesis reactions. Here, the compound may be bound to a solid support such as glass. The compound

represents one or more nucleic acid probes to which a target, *i.e.* the sample is bound. Such hybridisation experiments are disclosed in WO 95/00530.

The compound according to the invention may be used to distinguish single base mismatches. In U.S. Pat. 5,700,638 such experiments are described in Example 2. US Pat. 5,552,270 also describes such an approach. Here, the compound according to the invention comprises an oligonucleotide of defined sequence. An array is generated comprising numerous different sequences each suited to test a defined sequence.

The compound according to the invention may be used to analyse the expression of genes.

The compound according to the invention may be used to map genomes of organisms.

The compound according to the invention may comprise enzymatic functions as BM. In each case, this facilitates solid-support enzymatic reactions. In a preferred embodiment of the invention the compound is used for nucleic acid synthesis reactions. Here, the BM of the compound is preferentially a template dependent polymerase.

The inventors have found that the compound according to the invention gives unexpectedly good results when used on solid-phase in *in-vitro* DNA synthesis reactions (see also example 1 and Fig. 5).

Thus in a preferred embodiment of the above process at least one compound according to the invention is bound to a solid-support.

As a thermally stable polymerase, a DNA polymerase may be selected from the group comprising Taq DNA polymerase, Tth DNA polymerase or KlenTaq (Taq DNA polymerase (-exo5'-3'), Korolev et al., (1995) Proc. Natl. Acad. Sci. USA 92, 9246-9268. The use of Taq DNA polymerase in the method of the present invention is especially preferred.

Alternatively as a thermally stable polymerase, a DNA polymerase which has a decreased discrimination against the four ddNTPs with respect to wild-type Taq DNA polymerase in the buffer or under the conditions used for thermal cycling is preferred. More preferably, a DNA polymerase Taq polymerase carrying a "Tabor-Richardson" mutation or a functional derivative

thereof which also lacks 5'-3' exonuclease activity such as, for example, AmplitaqFS<sup>TM</sup> (Taq DNA polymerase (-exo5'-3')(F667Y), Tabor and Richardson (1995), loc. cit.), Taquenase<sup>TM</sup> (Taq DNA polymerase  $\Delta$ 235(-exo5'-3')(F667Y), Tabor and Richardson (1995), loc. cit.) and Thermo-Sequenase<sup>TM</sup> (Taq DNA polymerase (-exo5'-3')(F667Y), Tabor and Richardson (1995), loc. cit.) as well as mixtures thereof or other DNA polymerases and mixtures thereof which are thermally stable can be used in the process of the present invention. The use of Thermo Sequenase<sup>TM</sup> or any other DNA polymerase having a high ability to incorporate ddNTPs in the method of the present invention is especially preferred.

Alternatively as a thermally stable polymerase, a DNA polymerase which has a decreased discrimination against labeled nucleotide may be used.

The present invention, *i.e.* the process also provides for the use of two or more polymerases in the process or additional enzymes such as amplification enhancing reagents such as thermostable pyrophosphatase or enzymes which enhance the processivity of the polymerase such as PCNA (proliferating cell nuclear antigen) homologues. Enzyme mixtures may be equally applied.

The number of thermal cycles may range from about 1 to about 50 depending on the amount of template DNA and its purity. Generally, the inventors have found that very surprisingly extremely short cycles give good results. As the availability of the compound according to the invention is high in the process according to the invention the cycle period may be short, thus disadvantageous denaturing of proteins, *e.g.* the polymerase when in contact with glass occurs at a lower rate and the reaction may run efficiently without loss of function of enzyme.

Routinely, cycling consists of (i) a denaturing cycle, (ii) an annealing cycle and (iii) an extension cycle. Alternatively, only two cycles may be applied, (i) a denaturing cycle and (ii) an annealing and extension cycle.

Preferably the denaturing cycle is performed at between 100°C and 85°C, more preferably at between 98°C and 90°C, most preferably at between 96°C and 92°C.

Preferably the annealing cycle is performed at between 80°C and 45°C, more preferably at between 70°C and 50°C, most preferably at between 60°C and 55°C.

Preferably the extension cycle is performed at between 80°C and 50°C, more preferably at between 75°C and 60°C, most preferably at between 73°C and 68°C.

Preferably the denaturing cycle is performed for 3 minutes, more preferably for 30 seconds, most preferably for under 10 seconds.

Preferably the annealing cycle is performed for 3 minutes, more preferably for 30 seconds, most preferably for under 10 seconds.

Preferably the extension cycle is performed for 3 minutes, more preferably for 30 seconds, most preferably for under 10 seconds, however the extension time vary depending on the length of the template, in particular the extension time may be raised if the template length increases.

Buffers components which can be used can include, but are not limited to, Tris-HCl at a pH of about 7.0 to 10 and concentration of about 2 to 60 mM, ammonium sulfate at a concentration of about 10-20 mM, preferably 15 mM, MgCl<sub>2</sub> at a concentration of about 1 to 10 mM, and optionally, about 0.05 mM mercaptoethanol, about 0.28% Tween® 20 and/or about 0.02% Nonidet® 40.

Nucleotide triphosphates are preferably deoxynucleotides and can be selected from, but are not limited to, dGTP, dATP, dTTP and dCTP. In addition, derivatives of deoxynucleotides, which are defined as those deoxynucleotides which are capable of being incorporated by a thermally stable DNA polymerase into nascent DNA molecules synthesized in the thermal cycling reaction, can also be used according to the invention. Such derivatives include, but are not limited to thionucleotides, 7-deaza-2'-dGTP, 7-deaza-2'-dATP as well as deoxyinosine triphosphate which may also be used as a replacement deoxynucleotide for dATP, dGTP, dTTP or dCTP. The above mentioned deoxynucleotides and derivatives thereof are preferably used at a concentration of about 50 µM to about 4 mM.

Preferable the nucleotides are mixes of all four and at 200 µM per nucleotide.

In a preferred embodiment one or more of the nucleotides incorporated are labelled. For example, single and differential labels may consist of the group comprising enzymes such as β-galactosidase, alkaline phosphatase and peroxidase, enzyme substrates, coenzymes, dyes, chro-

mophores, fluorescent, chemiluminescent and bioluminescent labels such as FITC, Cy5, Cy5.5, Cy7, Texas-Red and IRD40(Chen et al. (1993), J. Chromatog. A 652: 355-360 and Kambara et al. (1992), Electrophoresis 13: 542-546), ligands or haptens such as biotin, and radioactive isotopes such as  $^3\text{H}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{125}\text{I}$  and  $^{14}\text{C}$ .

In one embodiment of the method of the invention, the nucleic acid molecule to be amplified can be present in the form of total genomic DNA, which is preferably in an uncloned or unpurified form. Preferably, the genomic DNA has a length greater than or equal to 2 kb. Generally all forms of template may be used, *e.g.* purified nucleic acids, *i.e.* nucleic acids where one fraction may be enriched or not, one example being plasmid DNA the other purified genomic DNA. The process may be suited for use with complex mixtures of DNA such being purified but not substantially fractionated genomic DNA or non-complex mixtures such being purified and substantially fractionated DNA *e.g.* plasmid DNA.

In a further preferred embodiment of the method of the invention, the nucleic acid molecule to be amplified can be present in the form of RNA. One polymerase or a mixture of two polymerases maybe utilized: a first DNA polymerase for example, Taq polymerase, and a second DNA polymerase with the capability to reverse transcribe RNA into DNA preferably Taq DNA polymerase (Jones et al., Nucl. Acids Res. 17: 8387-8388 (1989)) or Tth DNA polymerase (Myers et al., Biochemistry 30: 7666-7672 (1991)).

The invention also covers a kit for use in molecular biology or chemistry comprising at least the compound according to the invention. The kit may also comprise other reagents or enzymes such as buffers, nucleotides or the like. The kit may be used for diagnostics. Here, the kit may comprise a compound according to the invention or a compound according to the invention bound to a solid support, wherein the biomolecule moiety is represented by one or more oligonucleotides specific for particular *e.g.* genes or alleles.

While the foregoing has been set forth in detail, the Examples are presented for elucidation, and not limitation. Modifications and improvements on the compound and or process according to the invention disclosed above which are within the purview and abilities of those in the art are included within the scope of the claims.



**Example 1: (protein binding and detection):***Organo-silane derivatization:*

In order to introduce the organo-silane modification to the protein 10 $\mu$ l of purified BSA solution (10 $\mu$ g/ $\mu$ l, obtained from New England Biolabs Cat. No. 007-BSA) was co-incubated with 6 $\mu$ l EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide Hydrochloride, 100mg/ml, Sigma-Aldrich Cat. No. E1769), 10 $\mu$ l of an aqueous (5% v/v in 96% ethanol) solution of APTS (3-Aminopropyltrimethoxysilane, Sigma-Aldrich Cat. No. 28,177-8) and 24 $\mu$ l of MES (2-(N-Morpholino)ethanesulfonic acid, 100mM pH 5.5 (HCl), Sigma-Aldrich Cat. No. M2933) for 1 hour at ambient temperature.

*Attachment to glass-slides:*

The activated protein solution was spotted directly without further purification using a GMS 417 Arrayer (Genetic MicroSystems) at 1 hit per dot (corresponds to approximately 0.5 nl applied to the glass surface in a spot with a diameter of 180 $\mu$ m). After the spotting process the slides were washed twice with water and air-dried.

*Immuno-assay and detection:*

Specific detection of the immobilized BSA was accomplished by incubation with an anti-BSA monoclonal antibody (1:100 in 0.05% Tween, Sigma-Aldrich Cat. No. B2901) labelled with CY-3 fluorescent dye using the Mab Labeling Kit protocol obtained from Amersham Pharmacia Biotech (Cat. No. PA 33001). Following the 1 hour incubation at ambient temperature the slide was washed three times with 0.05% Tween and air-dried. Scanning of the fluorescent sample was accomplished in a fluorescent scanning device (Genetic MicroSystems, Array Scanner GMS 418). The results are shown in Fig. 1.

**Example 2: (Immobilization of protein molecules on untreated glass surfaces via indirect coupling to an NH<sub>2</sub>-silane moiety by EDC-mediated activation of COOH-groups)**

EDC is a popular carbodiimide that is widely used in reactions for conjugating biomolecules (Yamada, H., Imoto, T., Fujita, K., Okazaki, K. & Motomura, M. (1981) Selective modification of aspartic acid-101 in lysozyme by carbodiimide reaction. *Biochemistry*, 20, 4836-4842; Chase,

J.W., Merrill, B.M. & Williams, K.P. (1983) F sex factor encodes a single-stranded DNA binding protein (SSB) with extensive homology to *Escherichia coli* SSB. *Proc. Natl. Acad. Sci. U.S.A.*, 80, 5480-5484). Upon incubation with a biomolecule containing a carboxyl group, the latter is attacked by EDC to form a highly reactive, O-acylisourea intermediate. This active species can then react with a nucleophile such as a primary amine to form an amide bond (Williams, A. & Ibrahim, I.A. (1981) A mechanism involving cyclic tautomers for the reaction with nucleophiles of the water-soluble peptide coupling reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). *J. Am. Chem. Soc.*, 103, 7090-7095). Other nucleophiles such as sulfhydryl groups and oxygen atoms may act as attacking groups as well. However, the resulting products are unstable, whereas the reaction of EDC with a water oxygen (hydrolysis) represents a competing reaction in aqueous environment. Advantageously, sulfo-NHS (cf. Materials) can be added to the EDC-mediated coupling reaction to increase the stability of the active intermediate. The hydroxyl of sulfo-NHS reacts with the EDC active-ester intermediate forming a sulfo-NHS ester that enhances the stability of the activated carboxylate. As in the EDC-mediated reaction alone, the resulting product is a stable amide linkage.

Proteins are large biomolecules that contain a number of carboxyl (COOH) groups in their amino acid side chains (e.g. asp, glu) as well as on their carboxy-terminal end. Upon incubation with EDC or EDC/Sulfo-NHS one or more of these functional COOH-groups can be activated, ultimately forming a covalent linkage with the primary amine of APTS (cf. Materials) provided in the solution.

#### **Detailed description of the assay**

BSA (20 mg/ml) was added to a solution containing 20 mg/ml EDC, 0.6% APTS and 20 mM MES/pH 7.5 (2-Morpholinoethanesulfonic acid; SIGMA-ALDRICH). When sulfo-NHS was used the reaction concentration was adjusted to 10 mg/ml. The final reaction assay was mixed in conventional microtitre plates and incubated for 60' at room temperature with gentle shaking on an eppendorf incubator. Prior to the spotting process the plate and the prepared glass slides were transferred to a GMS 417 Arrayer (Affymetrix, former Genetic Microsystems). Immobilization was carried out at one hit per dot followed by a double washing step for 3 seconds in HPLC-purified water after each spotting step. Each protein-silane-EDC solution used was spotted in rows of 10 as a control of spotting quality (Fig. 2).

After the spotting process was complete, the slides were removed carefully from the spotting hood, transferred to a humid chamber and incubated over night at room temperature.

Following this over night incubation the slides were washed extensively in a 1% solution of SDS in HPLC-purified water for 15' in a glass container under vigorous stirring. Afterwards, the slides were rinsed with HPLC-purified water and dried by compressed air.

### **Immunoassay**

For detection of immobilized proteins on the glass surface fluorescence-labeled antibodies were diluted in 1x TBST (10 mM Tris/150 mM sodium chloride/0,5 % Tween-20/pH 8.0 adjusted with HCl). Various dilutions of antibody were assayed for their performance in the solid phase immunoassay. Working dilutions of the labelled antibody usually were in the range of 1:500 - 1:1000.

The assay was carried out by transferring the diluted antibody to the arrayed proteins on the glass surface and further incubation in a humid chamber for 60' at room temperature. Following the immunoassay the slide was rinsed briefly with HPLC-purified water and used for a pre-scan. For a washing step, the slide was transferred to a Falcon tube containing 1xTBST with vigorous shaking on an Eppendorf Incubator.

An example of such an immunoassay is depicted in Fig. 3. Three rows of a BSA/EDC/APTS solution have been spotted on untreated glass slides each represented by ten identical replicates (BSA). The latter demonstrates the homogeneity of spot morphology and assay quality in the immunological detection reaction carried out thereafter. As a control, an identical array of an APTS/EDC coupling reaction containing no BSA has been spotted in parallel (Control). The immunoassay using labeled anti-BSA antibody demonstrates that the silane-coupled BSA is retained on the glass surface and can be detected by a sensitive biological binding assay. Evidently, no background binding to the untreated glass surface has been observed and unspecific binding to the APTS/EDC mixture alone is negligible.

### **Materials**

Commercially available microscopy slides were immersed in a 1:1 solution of 1-methanol/hydrochloric acid for at least 12 hours at room temperature. The slides were rinsed in HPLC-purified water extensively, dried under compressed air and used for spotting immediately.

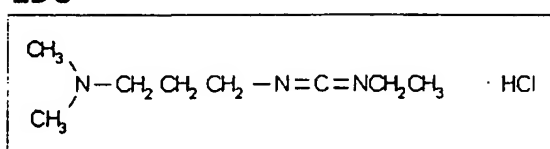
### 3-Aminopropyl-trimethoxysilane (APTS)

A 97% solution of APTS was purchased from SIGMA-Aldrich. A 5% working solution of was prepared by a dilution in HPLC-purified water. The solution can be stored at room temperature and should be stable at least one month.

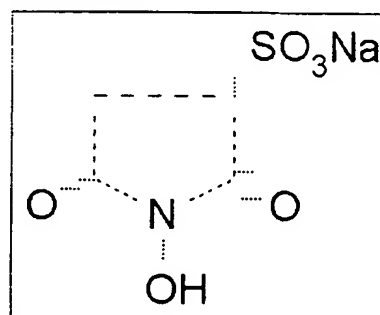
### 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; EDAC) & N-Hydroxysulfosuccinimide (sulfo-NHS)

EDC and sulfo-NHS were obtained from SIGMA-ALDRICH. A working solution of 100 mg/ml EDC was prepared by dissolving the according quantity of EDC powder in HPLC-purified water. Accordingly, a 100 mg/ml working solution of Sulfo-NHS was prepared in HPCL-purified water. Both solutions were freshly prepared prior to their use.

EDC



Sulfo-NHS



### Proteins and antibodies

Bovine serum albumin (BSA) was purchased from SIGMA-ALDRICH. BSA protein powder was dissolved in HPLC-purified water to obtain a 20 mg/ml concentration and stored at 4°C. Anti-bovine serum albumin antibody was purchased from SIGMA-ALDRICH. The antibody was

labeled with a commercially available Cy3 fluorescence dye from Amersham according to the manufacturer's protocol. The labelled antibody was stored at -20°C in aliquots of 100 µl.

### Figure Captions:

#### Fig. 1:

Fig. 1 shows the detection of a specific protein immobilized using the organo-silane derivatization protocol according to the invention. The figure shows the fluorescent image of the immunoassay specifically detecting immobilized BSA using a CY-3 labeled monoclonal anti-BSA antibody. The array contains 3 columns with 10 replicate spots each. The control spots contained no protein.

#### Fig. 2:

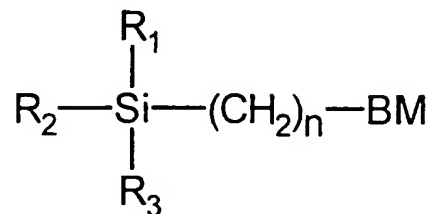
Fig. 2 shows an schematic outline of the immobilization of protein molecules on untreated glass surfaces *via* indirect coupling to an NH<sub>2</sub>-silane moiety by EDC-mediated activation of COOH-groups according to example 2.

#### Fig. 3:

Fig. 3 shows the results of an immunoassay for detection of immobilized proteins on the glass surface using fluorescence-labeled antibodies. Three rows of a BSA/EDC/APTS detection of a specific protein immobilized using the organo-silane derivatization protocol according to the invention. The figure shows the fluorescent image of the immuno-assay specifically detecting immobilized BSA using a CY-3 labeled monoclonal antibody.

1. Compound comprising a biomolecule moiety and an organo-silane moiety as represented in formula 1

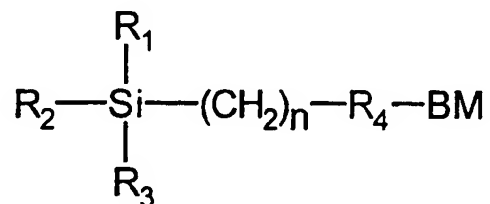
formula 1:



wherein,  $\text{R}_1$ ,  $\text{R}_2$ , and  $\text{R}_3$  are each and independently alkoxy groups and BM represents a biomolecule moiety selected from the group comprising one or more amino acids, peptides and proteins and derivatives thereof and wherein  $n$  is an integer from 0 to 18.

2. Compound according to claim 1, wherein the biomolecule moiety shows enzymatic activity, acts as a probe in molecular analysis or is the target of an enzymatic activity.
3. Compound according to claim 1 or 2, further comprising a linking moiety interposed between the organo-silane moiety and the biomolecule moiety where said compound is represented by formula 2

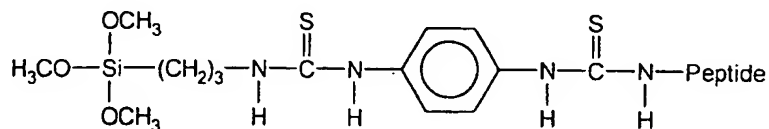
formula 2:



wherein,  $\text{R}_1$ ,  $\text{R}_2$ , and  $\text{R}_3$  are each and independently alkoxy groups, BM represents the biomolecule moiety or a derivative thereof,  $n$  is an integer from 0 to 18 and  $\text{R}_4$  represents the linking moiety.

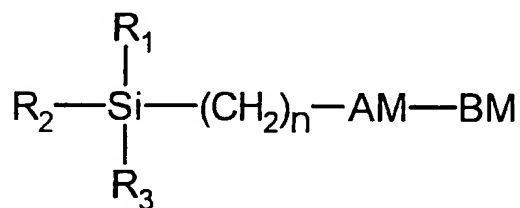
4. Compound according to any of claims 1 to 3, wherein  $R_1$ ,  $R_2$ , and  $R_3$  is each and independently a methoxy group.
5. Compound according to claim 4, wherein  $R_4$  is selected from the group comprising arylene(bisthiourea) and alkylene(bisthiourea).
6. Compound according to claim 5, wherein  $R_4$  is phenylenebisthiourea.
7. Compound according to claim 6, wherein the compound is represented by formula 4

formula 4:



8. Compound according to any of claims 3 to 7, wherein the compound further comprises an adapter moiety interposed between the organo-silane moiety and the biomolecule moiety where said compound is represented by formula 5,

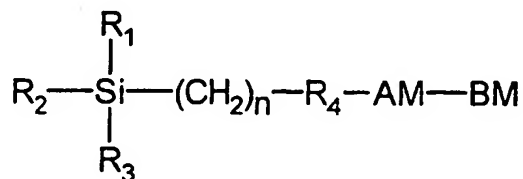
formula 5:



or alternatively

the compound further comprises an adapter moiety interposed between the linking moiety and the biomolecule moiety where said compound is represented by formula 5 A,

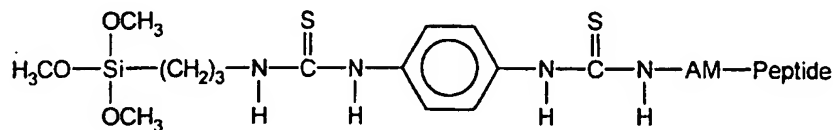
formula 5 A:



wherein,  $R_1$ ,  $R_2$ , and  $R_3$  are each and independently alkoxy groups,  
 BM represents the biomolecule moiety or a derivative thereof,  
 $n$  is an integer from 0 to 18,  $R_4$  represents the linking moiety and  
 AM represents the adapter moiety

9. Compound according to claim 8, wherein  
 the adapter moiety is selected from the group comprising  $-(CH_2)_n-$ ,  $-[(CH_2)_2O]_n-$  wherein  
 $n$  is an integer from 0 to 18.
10. Compound according to any of claims 1 to 9, wherein the biomolecule moiety is linked to the  
 organo-silane moiety via its N-terminal or its carboxy-terminal end.
11. Compound according to any of claims 3 to 9, wherein the biomolecule moiety is linked to the  
 linking moiety via its N-terminal or its carboxy-terminal end.
12. Compound according to claim 8 or 9, wherein the biomolecule moiety is linked to the  
 adapter moiety via its N-terminal or its carboxy-terminal end.
13. Compound according to claim 12, wherein the compound is represented by formula 6

formula 6:

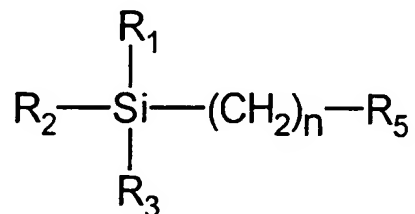


14. Compound according to any of the aforementioned claims, characterized in that the peptide  
 is an antibody or a functional part thereof.



15. Process for the synthesis of a compound according to any of claims 1 to 14, comprising reacting an organo-silane with a biomolecule moiety selected from the group comprising one or more amino acids, peptides and proteins and derivatives thereof, wherein the organo-silane is represented by formula 7:

formula 7:



wherein  $R_1$ ,  $R_2$ , and  $R_3$  are each and independently an alkoxy group,  $R_5$  is selected from the group comprising  $-NH_2$ ,  $-SH$ ,  $-COOH$ ,  $-PO_4$ ,  $-I$ , N-hydroxysuccinimidylester and  $n$  is an integer from 0 to 18.

16. Process according to claim 15, characterized in that the biomolecule is first reacted with an adapter molecule and subsequently reacted with the organo-silane.
17. Process according to claim 15, characterized in that the biomolecule is initially reacted with a linking molecule and subsequently reacted with the organo-silane,

or alternatively

the organo-silane is reacted with the linking molecule and subsequently reacted with the biomolecule,

wherein

the linking molecule is a bifunctional reagent.

18. Process for the synthesis of a compound according to any of claims 8 to 14, characterized in that,

- 25 -

(i) a biomolecule is reacted with an adapter molecule resulting in reaction product A;  
 reaction product A is reacted with a linking molecule resulting in reaction product B, and  
 reaction product B is reacted with an organo-silane

or alternatively

(ii) a biomolecule is reacted with an adapter molecule resulting in reaction product A;  
 a linking molecule is reacted with an organo-silane resulting in reaction product C and  
 reaction product A and C are reacted

or alternatively

(iii) an adapter molecule is reacted with a linking molecule resulting in reaction product D,  
 the reaction product D is reacted with the biomolecule resulting in reaction product B and  
 reaction product B is reacted with an organo-silane

or alternatively

(iv) an adapter molecule is reacted with a linking molecule resulting in reaction product D,  
 the reaction product D is reacted with an organo-silane resulting in reaction product E  
 and  
 reaction product E is reacted with a biomolecule.

19. Process according to claim 16 or 18, characterized in that the adapter molecule is represented  
 by formula 8,

formula 8:



wherein  $R_6$  is selected from the group comprising yanoethylphosphoramidites,

Z is selected from the group comprising -NH<sub>2</sub>, -SH, -PO<sub>4</sub>, -COOH, -I, and  
n is an integer from 0 to 18.

20. Process according to claim 17 and 18, characterized in that the linking molecule is selected from the group comprising arylenediisothiocyanate, alkylenediisothiocyanate, bis-N-hydroxy-succinimidylesters, hexamethylenediisocyanate and N-(γ-maleimidobutyryloxy)succinimide ester.
21. Process according to claim 20, characterized in that the linking molecule R<sub>4</sub> is phenylene diisothiocyanate.
22. Process for manufacturing a support comprising a compound according to any of claims 1 to 14, characterized in that the support is reacted with the organo-silane molecule having undergone any of the reactions of the processes according to any of claims 15 to 21.
23. Support, obtainable through the process according to claim 22.
24. Support, comprising a compound according to any of the claims 1 to 14, wherein the support exhibits a coating density of at least 1 pmol of biomolecule per mm<sup>2</sup>, preferably 10 pmol of biomolecule per mm<sup>2</sup> and most preferably 80 pmol of biomolecule per mm<sup>2</sup>.
25. Support according to claim 23 or 24, wherein the support is selected from the group comprising nitrocellulose, nylon, controlled-pore glass beads (CPG), polystyrene, activated dextran, modified polystyrene, styrene-acrylnitril-copolymers, polycarbonate, cellulose, polyamide and glass.
26. Use of a compound according to any of claims 1 to 14 in a nucleic acid synthesis reaction, in a primer extension reaction, in a reverse transcription reaction of RNA into DNA, in a nucleic acid hybridization reaction or a nucleic acid amplification reaction for analyzing the expression pattern of genes, for analyzing genotypes or alleles, for analyzing the expression pattern of proteins, in an antibody-specific reaction, as a probe in molecular analysis, as a target for enzymatic reactions or other enzymatic reactions.

27. Use according to claim 26, wherein the compound is bound to a solid support, more particularly a support according to any of claims 23 to 25.
28. Process for a nucleic acid synthesis reaction comprising the steps of
- i) combining at least one sample comprising one or more target regions with at least one nucleotide triphosphate, a polymerase, optionally a buffer and at least one compound according to any of claims 1 to 14 to form a reaction mixture,
  - ii) exposing the reaction mixture of step i) to at least one temperature cycle including at least a high temperature denaturation phase and a lower temperature extension phase, and thereby producing at least a partially amplified product.
29. Process according to claim 28, characterized in that the compound is bound to a solid support.
30. Kit, comprising a compound according to any of the claims 1 to 14.

BEST AVAILABLE COPY

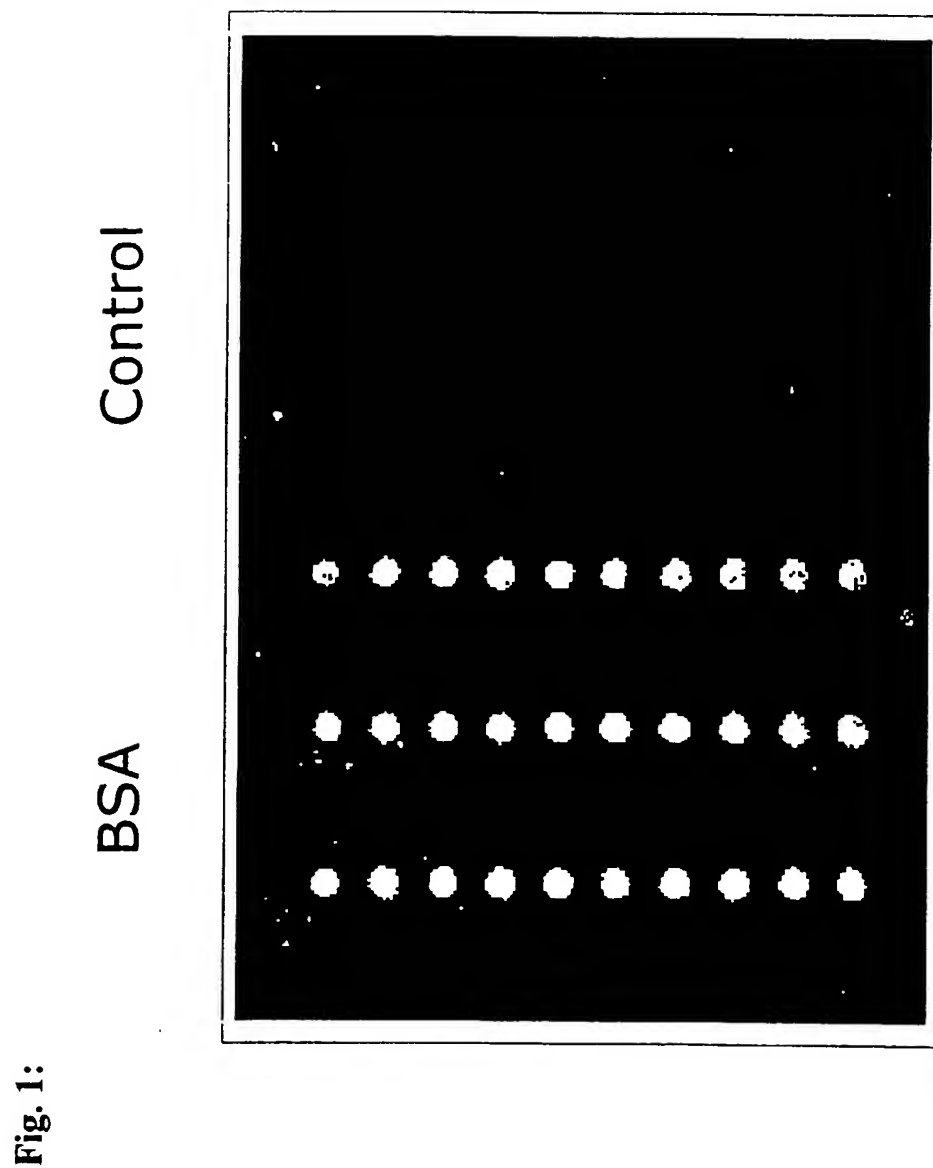
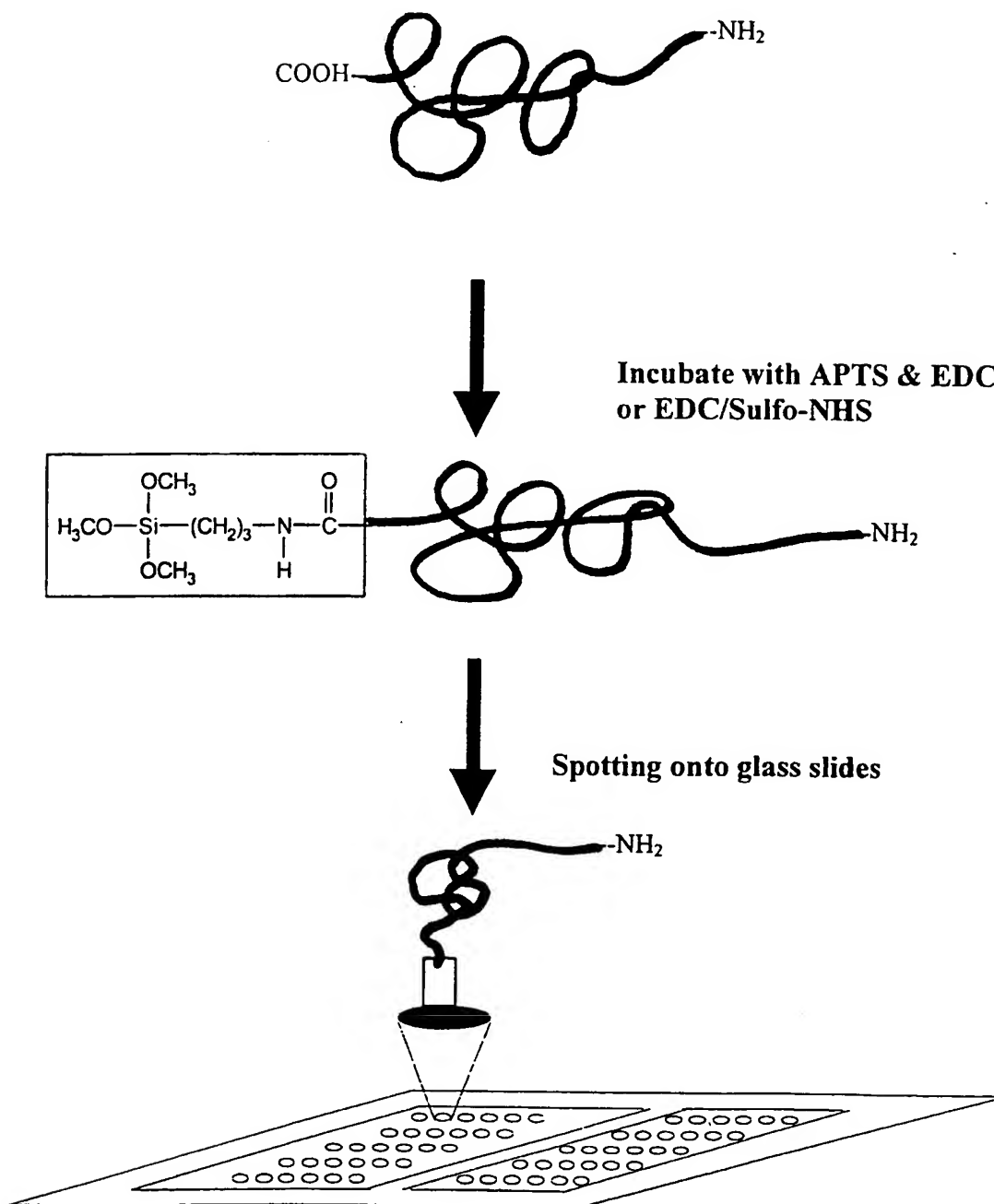


Fig. 2:

## Silane-modified proteins



**Fig. 3:**

**Direct detection of immobilized protein  
by a fluorescence (Cy3) labelled antibody**

